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# Proof of Concept to Isolate and Culture Primary Muscle Cells from Northern Elephant Seals to Study the Mechanisms that Maintain Aerobic Metabolism Under the Hypoxic Conditions of Breath-hold Diving

Shane B. Kanatous
Department of Biology
1878 Campus Delivery
Colorado State University
Fort Collins, Co 80523-1878
phone: (970) 491-0782 fax: (970) 491-0649 email: kanatous@colostate.edu

Award Number: N000141210895
http://extremephysiology.biology.colostate.edu

## LONG-TERM GOALS

To isolate and culture primary muscle cells from the swimming muscles of northern elephant seals.

## OBJECTIVES

To develop a protocol for the isolation and culturing of primary muscle cells from northern elephant seals.

## APPROACH

This work will be carried out in collaboration with Drs Daniel Costa, and Daniel Crocker. We will collect muscle biopsies from animals that are being sedated for their labs' respective projects. By utilizing the same animals and working with these collaborators, we will minize the impact and number of animals being handled at Año Nuevo State Reserve.

*Muscle sampling*
Biopsy samples of approximately 50 mg will be collected with a 6-mm biopsy cannula (Depuy, Warsaw, Indiana) from the swimming (M. longissimus dorsi) muscle. Before the biopsy is taken, the skin is first cleaned with betadine scrub. A small area the size of a dime is shaved and one ml of 2% Lidocaine is then injected beneath the skin around the biopsy site to minimize the trauma to the biopsy site. The skin is punctured with a #10 scalpel and the biopsy needle inserted to a depth of 3-4 inches (the blubber layer is typically 2 inches thick). Once collected, the biopsy will be dipped in 100% ethanol for sterilization then placed into culture media (F10 or F10 plus lipids) and stored on ice. After collection the biopsy site will be cleaned with betadine scrub. Slight pressure is applied to the biopsy site after flushing with 4x4 gauze pads to stop any minor bleeding.

The biopsy samples will then be shipped overnight on ice to the lab at Colorado State University for primary cell isolation and culturing.

# Report Documentation Page

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Primary Personnel: Shane B. Kanatous, Amber E. Schlater, Teresa Garcia and Caitlin Kielhorn

**WORK COMPLETED**

September 2012
We successfully sampled 10 juvenile animals over a two day period in September, 2012. The first major huddle to the study was to determine the effect of the overnight shipping of the viability of the samples and the ability to isolate cells from the biopsies. That huddle was met with tremendous success both media preps; the F10 and F10 +lipids produced cells from both the tissue samples and the transfer media (see representative picture below).

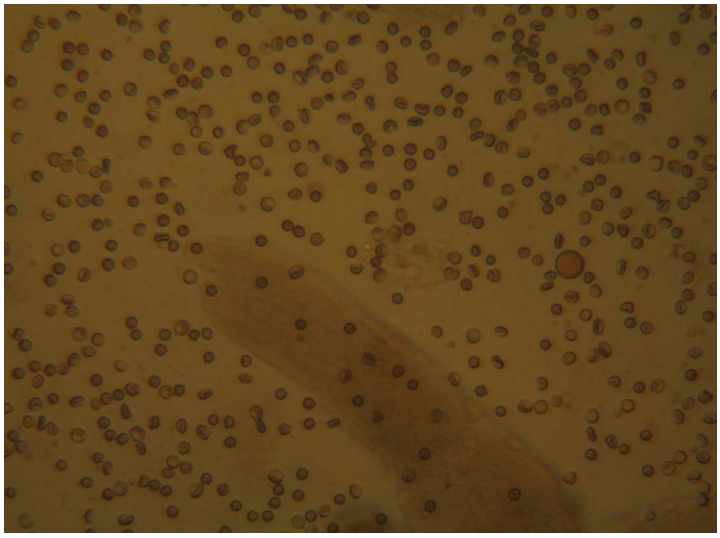

***Figure 1: Representative image of cells isolated from a skeletal muscle biopsy of a northern elephant seal. The first major huddle of the project was to determine if we would be able to obtain viable cells from the biopsies after being shipped overnight back to Colorado State University from the field site at Año Nuevo State Reserve, California. In the image numerous different cell types are seen floating above a piece of the muscle biopsy.***

Unfortunately, after initial cell ioslation we had some contamination issues during the proliferation stage. Those issues were presumed to be resolvable with the addition both antibiotics and antimycotics to the transfer and culturing medias. With those changes and our anticipated success in mind, plans were made to sample again in the late January or February 2013 timeframe.

January 2013
At the commencement of breeding season, we sampled 9 adult animals (8 males and 1 female). From these animals, we isolated viable myoblasts from each of the 10 individuals and subsequently froze down cells from 5 males and the female. With no appreciable difference in cell viability between F10 and F10+lipid medias, we decided to transfer cells exclusively in F10 (with antibiotics/antimycotics) for the next field trip.

February 2013
With newly-weaned pups available to sample, we successfully biopsied 21 pups. Viable cells were isolated from every sample and were eventually proliferated enough to freeze down cells from

numerous individuals. An abundance of fibroblasts, however, led us to incorporate pronase into the cell isolation technique for the next field trip.

April 2013
A second important step in this protocol was to verify the ability to re-grow cells from the frozen stock. In early April, we were able to re-grow cells isolated and frozen down from an adult male. Even more important for subsequent work was the ability to differentiate the myoblasts into mature myotubes.

Later in the monthy, we sampled 18 molting juveniles, of which myoblasts are proliferating from all 18 animals. At this time, it appears that we will have enough myoblasts to freeze cells from every animal within the next week.

We are planning an additional trip to the field in late May to collect samples and isolate cells from more adult females.

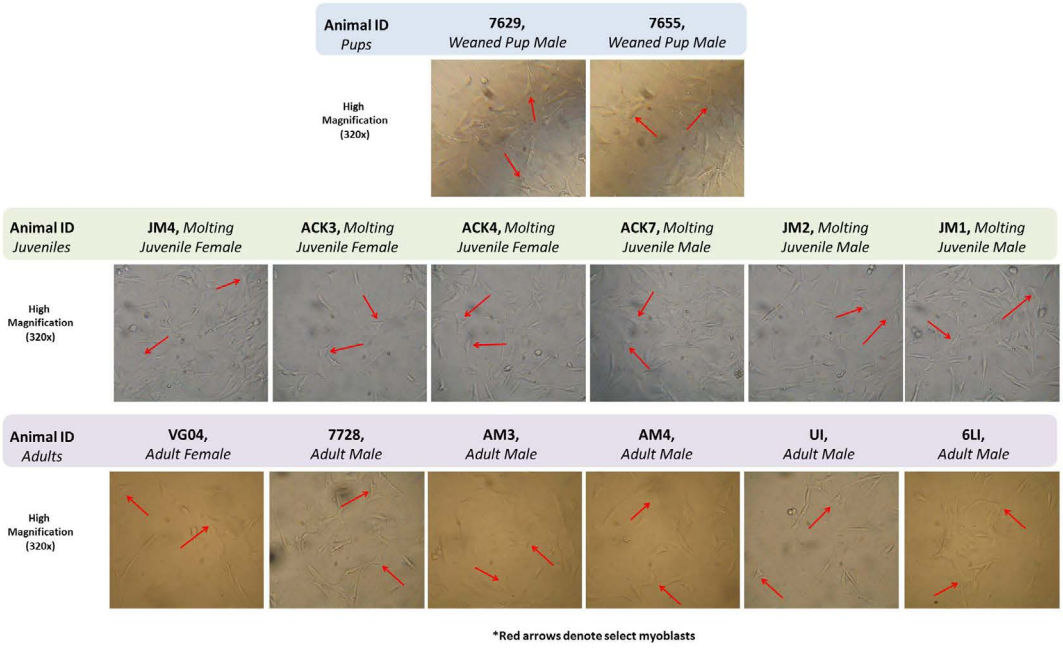


***Figure 2: Images of primary myoblasts obtained from biopsies of the swimming muscles of northern elephant seals. Select myoblasts are identified in each image by red arrows. The myoblasts can be distinguished from the fibroblasts by their distinct trianugular shape. After our first attempt in September 2012, we have been able to successfully isolate myoblasts from every animal. In addition, we have been able to proliferate enough cells to have frozen down stocks of cells from 6 adults, 16 juveniles and 2 pups.***

## RESULTS

### Isolation and growth of northern elephant seal primary muscle cells

The objective of this study was to establish a sampling technique and protocol for isolation primary muscle cells from the northern elephant seal. The technique for obtaining and shipping the samples is listed above in the methods section. After several experiments, the protocol for sample processing and culturing of the cells that elicited the greatest results is listed below.

*Satellite Cell Isolation Protocol*

1. Place biopsy on culture dish.
2. Add enough F10 based growth media to keep sample moist (250-500 microliters).
3. Under a dissecting microscope, remove as much tendon, fat, vessels, and connective tissue as possible; if possible, cut into two equal sized samples.
4. Cut biopsy with forceps in culture dish (do not mince – fragments that are too small have less effective results with mechanical trituration that follows digestion).
5. Move dish and sample into the cell culture hood (to maintain sterility).
6. Add 250µl of collagenase/dispase/CaCl2 OR pronase solution to tissue
7. Transfer minced tissue to sterile tube and incubate at 37$^{o}$C for 60 min (until mixture is a fine slurry).
8. During those 60 mins, gently swirl tube every 15 min.
9. In the hood, transfer the tissue and media to a 15 ml conical tube.
10. Spin down via low-speed centrifugation at 400xg for 5 minutes.
11. Remove supernatant and resuspend pellet (in same tube) in 5 ml of F10 based growth media.
12. FIRST TRITURATION: pass muscle fragments through 10ml glass pipette until tissue bits pass easily through tip.
13. Allow suspension to settle in 15ml conical tube so that remaining larger bits separate from the supernatant that contains the released cells.
14. Collect supernatant and transfer to new 15ml conical tube.
15. SECOND TRITURATION: add 5ml of F10 based growth media to 15 ml conical tube containing the remaining muscle pieces and replace the muscle trituration process
16. Allow second trituration to settle and collect supernatant in same 15ml conical tube with first trituration's supernatant.
17. Place a 40micrometer cell strainer onto a 50ml conical tube
18. Using a 10ml pipette, transfer the pooled supernatants from the two triturations to the 40 micrometer cell strainer

    *Carefully tap the side to reduce residual large debris from cell suspension
19. Add an additional 1ml F10 based growth media to drip through strainer to recover residual cells trapped by debris in the strainer

20. Centrifuge strained cells at 1000xg for 10 min
21. Carefully discard supernatant and resuspend pellet in 2 ml of f-10 based growth media + 0.5% lipid (prewarmed to 37$^{o}$C)
22. Plate cells on matrigel-coated plates for initial growth phase.
23. Check on cells in 24 hours.

After initial growth, the cells are transferred into standard growth media supplemented with 2.5% lipid (De Miranda et al., 2012) and plated onto gelatin-coated plates for proliferation. During this stage the cells were allowed to proliferate and frozen in fetal bovine serum with 10% DMSO or differentiated in standard differentiation media supplemented with 2.5% lipid to form myotubes (De Miranda et al., 2012). Up to date, we have frozen cells from 5 adult males, 1 adult female, 2 pups and 14 juveniles. Moreover, cells from these aforementioned frozen myoblast stocks can be thawed, plated, regrown, and differentiated into myotubes.

In summary, we have successfully isolated and grown primary muscle cells from all age classes of northern elephant seals. In addition, we have been able to freeze down multiple samples from each age class and subsequently regrown and differentiated the cells from the frozen stocks.

**Mitochondrial function in northern elephant seals (*Mirounga angustirostris*)**
Modern molecular biology techniques allow for detailed assessments of functionality within the intact mitochondrial network, the energetic powerhouse of eukaryotic cells. Specific functions of the electron transport system such as oxidative phosphorylation capacity, leak relative to specific complexes, mitochondrial integrity, substrate preference, and overall mitochondrial density can be characterized by providing various substrates to permeabilized muscle fibers and measuring changes in oxygen consumption with an oxygen respirometer. These measurements can subsequently tell us vital physiological information such as metabolic efficiency, heat generation, and aerobic capacity within the subcellular environment of a wide range of eukaryotic model systems.

In order to better assess aerobic respiration in adult male northern elephant seals (*Mirounga angustirostis*), an oxygen-limited athletic model, mitochondrial respiration was examined in muscle fibers of the primary swimming muscle (*M. longissimus dorsi*). Biopsies were taken with a 6mm biopsy cannula and muscle fibers were permeablized, allowing various metabolic substrates to flow freely to the mitochondria while oxygen flux was measured. Mitochondrial respiratory function was determined in permeabilized muscle fibers by high-resolution respirometry using a variety of electron transport chain, uncoupler, and inhibitor combinations.

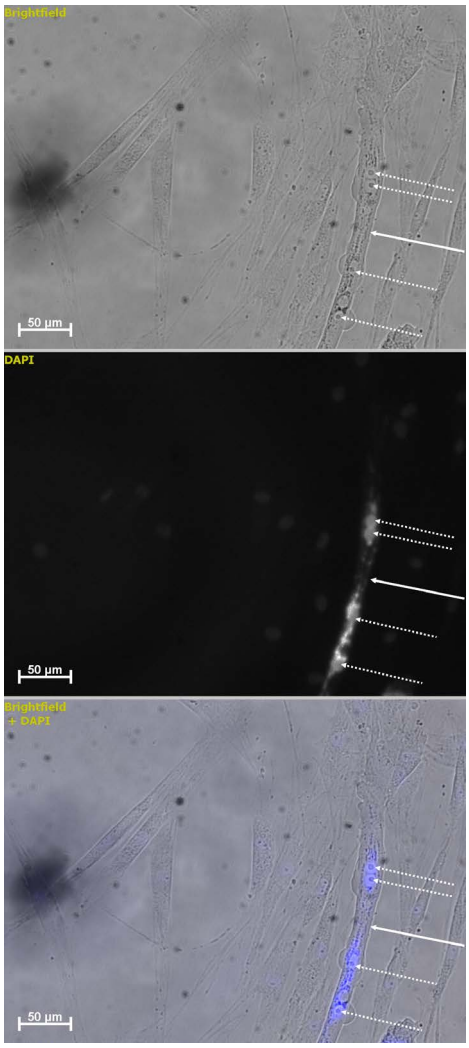


***Figure 3: A composite of a 7-day myotube from the nothern elephant seal. The solid arrow points out one of the multinucleated myotubes in the frame. The dashed arrows point to the nuclei. The top panel is a brightfield image, while the second panels shows the DAPI stained nuclei and the final panel is an overlay of the DAPI stained nuclei on the brightfield image.***

Preliminary adult male mitochondrial respirometry data (n=2) suggest that seals have 49% less total mitochondrial respiratory capacity compared to humans, with a higher capacity for fatty acid oxidation in the seals. In addition, inner mitochondrial membranes are extremely leaky to protons in seals (82% leak in seals, compared to 40% leak in humans); yet despite this leakiness, elephant seal mitochondria are more efficient at oxidative phosphorylation (i.e., making ATP) than humans. These two processes (the efficiency of ATP production and extent membrane proton "leak") were previously believed to be inversely proportional in mammalian mitochondria, such that increases must be mutually exclusive. However, because leak across the inner mitochondrial membrane is known to generate heat, these data coincide with the physiology of this endothermic diving mammal, which makes long distance migrations in cold water with a very thin blubber layer. Moreover, during these breath-hold exercise bouts, apnea and peripheral vasoconstriction limit oxygen and substrate delivery; thus, increased oxidative phosphorylation efficiency allows these animals to maximally utilize a finite supply of metabolites while maintaining aerobic respiration. These are the first mitochondria that have been

measured with an extreme capacity for leak without concomitantly compromising oxidative phosphorylation but rather an *increase* in capacity for oxidative phosphorylation.

In addition to mitochondrial respiration measures from adult male elephant seals, mitochondrial function was also evaluated in both male and female pups that were early in their post-wean fast (PWF). Interestingly, access to both sexes for this age class allowed us to establish a distinct difference between males and females regarding inner mitochondrial membrane leak. Specifically, female pups (n=5) early in their PWF had 47% more leak than male pups (n=3) early in their PWF. This unexpected disparity where female pups have a higher capacity for leakiness, or heat generation, across the inner mitochondrial membrane parallels physiology associated with projected fasting trends in these animals. Throughout the duration of fasts, male elephant seals have been shown to have more reliance on lipid-fueled metabolism from the liberation of stored fats, thus sparing their lean mass; in support of this phenomenon, addition of lipid substrate to these respiring mitochondria show these male pups as having the capacity to metabolize more lipid than females (53% more capacity for lipid-based oxidative phosphorylation). Fasting females, alternatively, have been shown to spare fat reserves and readily breakdown lean mass for gluconeogenesis, thus leaving them with less lean mass and more fat mass (Field et al 2005; Kelso et al 2012). As such, fasting females with less metabolically active lean mass are likely more thermally challenged than fasting males that retain their metabolically active lean mass. These preliminary data, therefore, suggest that fasting females may be compensating for this potential increased thermal challenge by increasing their leak and subsequent heat generation across the inner mitochondrial membrane.

## CONCLUSIONS

Adult northern elephant seals have lower muscle respiratory capacity compared to humans (per mg muscle tissue), but this varies with age and season. Despite lower respiratory capacity, elephant seal muscle has a similar capacity for oxidative phosphorylation (oxphos) using lipid substrate (palmitoylcarnitine + malate) as human muscle per mg tissue.

Maximal phosphorylation control of respiration using lipid and CHO substrates (RCR; i.e., the ability of ADP to drive oxphos) is similar in seals and human. However, max oxphos rates relative to respiratory enzyme capacity (SUIT1 P/E; an index of oxphos "efficiency") is higher in seals vs. humans in the presence of lipid substrate. This means that seals achieve oxphos rates that are closer to their maximal ETS enzymatic capacity compared to humans.

Despite similar phosphorylation control and greater oxphos efficiency compared to humans, seal muscle mitochondria have 60-80% greater capacity for respiratory leak in the presence of ADP and ATP, indicating a dramatically greater capacity to generate heat while maintaining highly efficient aerobic ATP production. In addition, seal mitochondria maintain oxidative efficiency with less available oxygen (approximately 65% less) then human mitochondria in the absence of myoglobin. This remarkable phenotype is unprecedented in mammalian biology, and appears to vary between sexes, age and season, indicating role for both genetic and environment queues.

## IMPACT/APPLICATIONS

**Significance:** We have established a reliable protocol for the isolation and growth of primary muscle cells from the northern elephant seal. This only the second diving mammal species from which primary muscle cells have been isolated and been able to be frozen, subsequently regrown and

differentiated. This protocol and unique characteristics of the muscle cells will allow future experiments to decipher the regulation of oxygen metabolism and its effects on development and animal performance which is of vital importance to understanding the physiological plasticity of organisms in a changing environment. The results of these future studies will determine whether the regulation of the expression of myoglobin is determined more by evolutionary history or environmental stimuli.

**Implications and application beyond our current research**: The techniques being developed here will have broad applications to other non-model organisms which cannot easily be exposed to different experimental conditions. The use of primary cells can be expanded to other cell types that could be obtained safely from animals using biopsy techniques. In addition, an unexplored area that could evolve from this technique is to expose the cultured cells to other environmental stresses such as the different aspects of climate change (i.e. temperature, salinity, pH, oxygen and carbon dioxide tensions), changes in substrate utilization or exposure to different contaminants to determine their effect on growth and development.